

## Identification of a Region of RyR1 That Participates in Allosteric Coupling with the $\alpha_{1S}$ (Ca<sub>v</sub>1.1) II–III Loop\*

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In skeletal muscle, excitation-contraction (EC) coupling and retrograde signaling are thought to result from direct interactions between the ryanodine receptor (RyR1) and the  $\alpha_1$  subunit of the dihydropyridine receptor ( $\alpha_{1S}$ ). Previous work has shown that the s53 region of  $\alpha_{1S}$  (residues 720–765 in the II–III loop) and regions R10 (1635–2636) and R9 (2659–3720) of RyR1 are involved in this signaling. Using the yeast two-hybrid system, we here report an interaction between s53 and the sR16 region of RyR1 (1837–2168, within R10), whereas no interaction was seen using upstream residues of the  $\alpha_{1S}$  II–III loop (s31, 666–709). The specificity of the s53-sR16 interaction was tested by using fragments of the cardiac RyR (RyR2) and DHPR ( $\alpha_{1C}$ ) that correspond to sR16 and s53, respectively. No interaction was observed for sR16 × c53 ( $\alpha_{1C}$  850–897), but weak interaction was occasionally observed for s53 × cR16 (RyR2 1817–2142). To test the functional significance of the s53 × sR16 interaction, we expressed in dyspedic myotubes a chimeric RyR (chimeraR16) in which sR16 was substituted for the corresponding region of RyR2. ChimeraR16 was found to mediate weak skeletal-type EC coupling. To test the necessity of sR16 sequence for coupling, we used “chimeraR16-rev,” in which sR16 and a small upstream region of RyR1 were replaced by RyR2 sequence. ChimeraR16-rev did not differ from RyR1 in its ability to mediate EC coupling. Thus, interaction between residues 720–765 of  $\alpha_{1S}$  and residues 1837–2168 of RyR1 appears to contribute to but is not essential for EC coupling in skeletal muscle.

In muscle cells, excitation-contraction (EC)<sup>1</sup> coupling is the process by which depolarization of the plasma membrane produces the large transient release of Ca<sup>2+</sup> from the sarcoplasmic reticulum, which in turn triggers contraction. Dihydropyridine receptors (DHPRs), L-type Ca<sup>2+</sup> channels in the plasmalemma, serve as the voltage sensors for EC coupling (1) and activate

ryanodine receptors (RyRs), intracellular Ca<sup>2+</sup> release channels in the sarcoplasmic reticulum membrane (2, 3). In cardiac muscle, entry of Ca<sup>2+</sup> through the DHPR activates the RyR (4). In skeletal muscle, however, entry of Ca<sup>2+</sup> through the DHPR is not required for EC coupling (5). Instead, a voltage-dependent conformational change in the II–III loop of the skeletal muscle DHPR  $\alpha_1$  subunit ( $\alpha_{1S}$ ) has been hypothesized to allosterically activate the skeletal muscle ryanodine receptor, RyR1. In addition to this orthograde EC coupling signal, which is transmitted from  $\alpha_{1S}$  to RyR1, there is also a retrograde signal whereby the presence of RyR1 increases the Ca<sup>2+</sup> current density produced by  $\alpha_{1S}$  (6).

Because the skeletal muscle isoforms of DHPRs and RyRs are required for skeletal-type EC coupling and retrograde signaling, functional expression of skeletal-cardiac chimeras of both proteins has been used to determine regions of  $\alpha_{1S}$  and RyR1 that participate in bi-directional signaling. Expression of chimeric DHPR  $\alpha_1$  subunits in dysgenic muscle (which lacks  $\alpha_{1S}$ ) has revealed that  $\alpha_{1S}$  residues 720–765 in the intracellular loop between domains II and III (the II–III loop) are necessary for bi-directional signaling in dysgenic myotubes (7, 8). Similarly, expression of chimeric RyRs in dyspedic myotubes (which lack RyR1) has shown that regions R9 (residues 2659–3720) and R10 (residues 1635–2636) of RyR1 participate in signaling interactions with  $\alpha_{1S}$  (9).

Despite these functional data, there is little evidence as to whether signaling between  $\alpha_{1S}$  and RyR1 results from direct contact between the two proteins. A number of studies have addressed this question *in vitro*. Direct binding in protein affinity columns has identified interactions between peptides corresponding to the II–III (10) and III–IV (11) loops of  $\alpha_{1S}$  and a peptide corresponding to residues 951–1112 of RyR1 (but not to the equivalent RyR2 residues). However, replacement of this region of RyR1 with the equivalent RyR2 residues in chimeric RyRs does not interfere with bi-directional interactions in functional assays (9). The responses of isolated RyRs to peptides corresponding to specific regions of DHPRs have also been used to study interactions between  $\alpha_{1S}$  and RyR1. In particular, residues 671–690 of  $\alpha_{1S}$  (“peptide A”) or subdivisions of this region have been found to activate RyR1 (12–18). However, skeletal sequence in this region is not required for EC coupling by intact DHPRs expressed in dysgenic myotubes (7, 8, 19, 20). The divergent results of functional and biochemical studies are difficult to reconcile and may indicate that there are multiple sites of contact, individually of low affinity, that mediate signaling between  $\alpha_{1S}$  and RyR1.

In this paper, we have attempted to identify domains of  $\alpha_{1S}$  and RyR1 that participate in *both* direct binding and functional coupling between the two proteins. Using the yeast two-hybrid system, we have observed a weak interaction between the s53

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<sup>1</sup> The abbreviations used are: EC, excitation-contraction; DHPR, dihydropyridine receptor; RyR, ryanodine receptor.

region of the  $\alpha_{1S}$  II–III loop (residues 720–765) and the sR16 region of RyR1 (residues 1837–2168 within R10). The functional significance of this interaction was tested using chimeric RyRs expressed in dyspedic myotubes. When substituted into a RyR2 backbone, region sR16 was sufficient to confer weak skeletal-type EC coupling (“chimeraR16”). The mirror chimera (“chimeraR16-rev”), in which sR16 and an upstream region of RyR1 were replaced by RyR2 sequence, was used to test whether skeletal sequence in the R16 region was required for bi-directional signaling. ChimeraR16-rev did not differ significantly from RyR1 in its coupling with  $\alpha_{1S}$ . These results suggest that R16 is one site of contact between RyR1 and DHPs that participates in EC coupling and that other such sites likely exist.

#### EXPERIMENTAL PROCEDURES

**Molecular Biology and Yeast Two-hybrid Assays**—For yeast two-hybrid interaction tests, portions of the rabbit skeletal muscle ryanodine receptor (RyR1) (21) or cardiac muscle ryanodine receptor (RyR2) (22) were inserted in-frame into the GAL4 activation domain plasmid pGAD424 (CLONTECH, Palo Alto, CA) by means of restriction sites added to the ends of the fragments generated by PCR. Similarly, dihydropyridine receptor  $\alpha_{1S}$  (23),  $\alpha_{1C}$  (24, 25), or  $\beta$  subunit (26) fragments were inserted in-frame into the GAL4 binding domain plasmid, pGBT9 (CLONTECH). Two fragments (sR16 and s53) were expressed and assayed for yeast two-hybrid interaction in both pGAD424 and pGBT9. All inserts generated by PCR were confirmed by automated DNA sequencing (Macromolecular Resources, Fort Collins, CO). For yeast two-hybrid clones, the template used, the amino acids expressed (in parentheses), and the primer pairs used were as follows: R10A (RyR1 1633–1858), 5'-GGAATTCCTCCGTGACCATGATGGCG-3', 5'-CGGGATCCAT-CCTCATCCCAAAGATGC-3'; R16 (RyR1 1837–2168), 5'-GCGAATTCAGTTCGTCGCGGTGCTCAAG-3', 5'-GCGGATCCGAGCGGCATC-TTGCGCAACTCGTT-3'; R10B (RyR1 2165–2414), 5'-GGAATTCGTGCTCATCGTGCAGATGG-3', 5'-CGGGATCCTTCTCAGAGTCCCTC-TCCCC-3'; R10C (RyR1 2408–2636), 5'-GGAATTCGAGGAGCCCCCT-GAAGAAAAC-3', 5'-CGGGATCCGAACTCGTTCCAGATGGGC-3'; R9A (RyR1 2636–2956), 5'-GGAATTCCTCGCCAAGATGCCGCTCA-3', 5'-CGGGATCCGGCGAACCGCTTCTCAATGGA-3'; R9B (RyR1 2950–3252), 5'-GGAATTCCTCCATTGAGAAGCGGTTCCGC-3', 5'-cggg-atcctgtcgcaTGAGCCGATCCAG-3'; R9C (RyR1 3246–3552), 5'-GGAA-TTCTGGATCCGGTCCATGGCAGAC-3', 5'-CGGGATCCAAATTCCTCC-GGACTCCTCGTC-3'; cR16 (RyR2 1817–2142), 5'-GCGAATTCCTCT-TGTGCTTCATC-3', 5'-CGGGATCCCATAAAGCTTCTCTCTCTCT-TT-3'; s31 ( $\alpha_{1S}$  666–709), 5'-GGAATTCGAGGCGGAGAGCCTGACTT-CC-3', 5'-CGGGATCCGGGCTTCTGCTCCAGCTTCTT-3'; s53 ( $\alpha_{1S}$ , 719–766), 5'-GGAATTCAGCTCAAGGTCGATGAG-3', 5'-CGGGATC-CTTGAGCTGCAGTCCGGC-3'; c53 ( $\alpha_{1C}$  850–897), 5'-GGAATTCAG-ATCAACATGGATGAC-3', 5'-CGGGATCCTTAAGGTGCAGCTCGGA-G-3'; s31 ( $\alpha_{1S}$  666–709), 5'-GGAATTCGAGGCGGAGAGCCTGACTT-C-3', 5'-CGGGATCCGGGCTTCTGCTCCAGCTTCTT-3'; s53 ( $\alpha_{1S}$ , 719–766), 5'-GGAATTCAGCTCAAGGTCGATGAG-3', 5'-CGGGATC-CTTGAGCTGCAGTCCGGC-3'; c53 ( $\alpha_{1C}$  850–897), 5'-GGAATTCAG-ATCAACATGGATGAC-3', 5'-CGGGATCCTTAAGGTGCAGCTCGGA-G-3';  $\alpha$ 1D ( $\alpha_{1S}$ , 342–382), 5'-GGAATTCGAGAAGGCCAAGTCCAGGG-GAACC-3', 5'-CGGGATCCTCAGGTCCTCCACGTCCATGAC-3'; and  $\beta$ 1D ( $\beta_{1A}$  151–330), 5'-GGAATTCAGGAGGGCTGCGAGGTT-3', 5'-CGGGATCCTGCTGGGGTTGTTGAGGA-3'.

Interactions between portions of DHPs and RyRs were assayed by expression of GAL4 activation and binding domain fusion proteins in the yeast strain HF7c using the Matchmaker Yeast Two-Hybrid kit according to manufacturer's protocols (CLONTECH). Pooled colonies collected from SD/–Trp/–Leu plates were spread on SD/–Trp/–Leu/–His plates and examined for the appearance of colonies after incubation for 4–10 days at 30 °C. Each transformation was repeated 5–15 times.  $\beta$ -Galactosidase activity was determined qualitatively by the appearance of a blue substrate in a filter lift assay.

The mammalian expression plasmid chimeraR16 was constructed by substituting nucleotides 5508–6462 of RyR1 for nucleotides 5449–6354 of RyR2 using *Eco*RI and *Xho*I restriction sites. This produced a chimeric ryanodine receptor that is RyR2 in sequence except for replacement of residues 1817–2118 by the residues 1837–2154 of RyR1. The plasmid encoding chimeraR16-rev was constructed by ligating a PCR fragment comprised of RyR2 nucleotides 4912–6358 into the *Age*I (4936) and *Xho*I (6466) sites in pCIneoRyR1 (6). This resulted in a

chimeric ryanodine receptor that had RyR1 sequence except for replacement of residues 1645–2154 by RyR2 residues 1637–2118.

**Expression of Ryanodine Receptors in Dyspedic Myotubes**—Primary cultures of myotubes were prepared from newborn dyspedic mice as previously described (27). Approximately 1 week after plating, plasmids carrying cDNA for RyR1, RyR2, chimeraR16, or chimeraR16-rev (0.3  $\mu$ g/ $\mu$ l) were microinjected along with cDNA encoding either the CD8 antigen (0.2  $\mu$ g/ $\mu$ l) (28) or green fluorescent protein (0.04  $\mu$ g/ $\mu$ l) (29) into single nuclei. Injected myotubes were identified either by binding of CD8 antibody-coated beads (Dynabeads M-450; DYNAL AS, Oslo, Norway) or by green fluorescence 48–72 h after injection. Expressing cells were examined for voltage-sensitive  $Ca^{2+}$  currents, the ability to contract in response to electrical stimulation, and intracellular  $Ca^{2+}$  transients.

Macroscopic  $Ca^{2+}$  currents and intracellular  $Ca^{2+}$  transients were measured simultaneously (30) using borosilicate glass patch pipettes, which had resistances of 1.5–2.0 M $\Omega$  when filled with an internal solution containing 145 mM cesium glutamate, 2 mM Cs<sub>2</sub>Cl, 8 mM MgATP, 10 mM EGTA, 10 mM HEPES, and 0.5 mM K<sub>5</sub>Fluo-3 (Molecular Probes, Eugene, OR) (pH 7.4 with CsOH). The composition of the standard extracellular solution was 10 mM CaCl<sub>2</sub>, 145 mM tetraethylammonium chloride, 0.003 mM tetrodotoxin, and 10 mM HEPES (pH 7.4 with tetraethylammonium hydroxide). The voltage clamp command sequence was to step from a holding potential of –80 mV to –30 mV for 1 s, to –50 mV for 30 ms, to the test potential for 200 ms, to –50 mV for 125 ms, and then back to the holding potential. Control currents elicited by 20–40 mV hyperpolarizations from the holding potential were used to measure linear capacitance (cells > 800 picofarads were excluded from further analysis) and to correct test currents for linear components of leakage and capacitive currents by means of digital scaling and subtraction. The cells were examined for their ability to contract in response to electrical stimulation (90–100 V, 5–10 ms). For electrically evoked contractions in the absence of  $Ca^{2+}$  entry through  $\alpha_{1S}$ , the cells were bathed either in Rodent Ringers in which the  $Ca^{2+}$  had been replaced by Mg<sup>2+</sup> (145 mM NaCl, 5 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM HEPES, pH 7.4 with NaOH) or, for chimeraR16-rev, in tissue culture medium (Dulbecco's modified Eagle's medium; Sigma) containing 0.5 mM CdCl<sub>2</sub> and 0.1 mM LaCl<sub>3</sub>. All data are presented as the means  $\pm$  S.E.

#### RESULTS

**Domain sR16 of RyR1 Binds to Domain s53 of  $\alpha_{1S}$** —To test the hypothesis that skeletal-type EC coupling results from a direct physical interaction between the II–III loop of  $\alpha_{1S}$  and a discrete domain of RyR1, we used the yeast two-hybrid system to test for interaction between regions of the two proteins that were previously identified in functional studies. In yeast, we expressed two regions of the  $\alpha_{1S}$  II–III loop as fusion proteins with the binding domain of the GAL4 transcription factor: s53 ( $\alpha_{1S}$  residues 719–767) and s31 ( $\alpha_{1S}$  666–709). We also expressed, as fusion proteins with the activation domain of GAL4, subdivisions of the R9 and R10 regions of RyR1 that were previously identified as important for signaling interactions with  $\alpha_{1S}$  (9). These subdivisions were ~300 residues in length, the maximum size that can be tested using the two-hybrid system. The skeletal constructs are indicated in Fig. 1 and are designated with the prefix “s.” To determine the specificity of interactions between RyR1 and  $\alpha_{1S}$ , we also tested corresponding regions of  $\alpha_{1C}$  and RyR2 for interactions; the cardiac constructs are designated with the prefix “c.” c53 ( $\alpha_{1C}$  residues 850–897) corresponds to s53, and cR16 (RyR2 1817–2142) corresponds to sR16.

Yeast co-transformed with pairs of constructs indicated in Fig. 1 were plated onto nutritional selection medium (SD/–Trp/–Leu/–His). Colonies (which indicate an interaction between the test fragments) were never observed for yeast co-transformed with s31 and any of the RyR constructs. This absence of colonies suggests that the s31 region does not interact with the RyR domains tested and is consistent with experiments using expression in myotubes demonstrating that this region of  $\alpha_{1S}$  is not critical for signaling interactions with RyR1 (7, 19, 20). In contrast, colonies were consistently observed for yeast co-transformed with sR16 and s53 ( $n = 15$ ), although their appearance

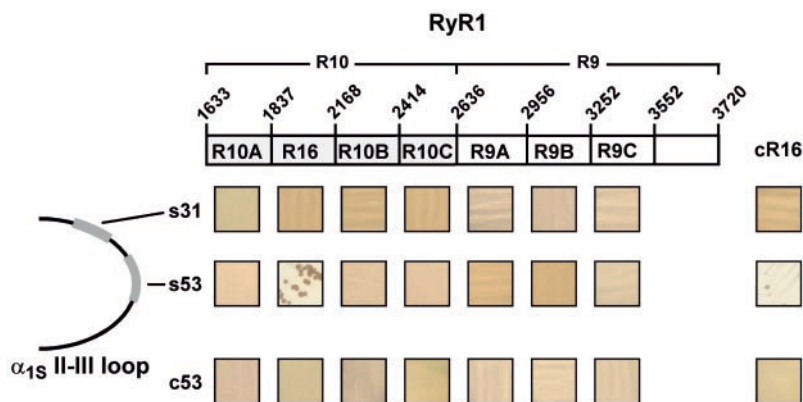


FIG. 1. The s53 region of the  $\alpha_{1S}$  II–III loop interacts specifically with the sR16 region of RyR1. Segments s31 and s53 (7) of the  $\alpha_{1S}$  II–III loop and segments R10 and R9 (9) of RyR1 are illustrated schematically at the left and top, respectively. For the yeast two-hybrid assays, the R10 and R9 regions of RyR1 were subdivided into smaller segments, with boundaries as indicated (see “Experimental Procedures” for additional details). Representative sections ( $\sim 2 \text{ cm}^2$ ) are shown of SD/–Trp/–Leu/–His nutritional selection medium plated with yeast strain HF7c co-transformed with pairs of segments from  $\alpha_{1S}$  and RyR1, as indicated; in the bottom row, c53 (residues 851–896 of  $\alpha_{1C}$ ) was used, and in the far right column cR16 (residues 1819–2142 of RyR2) was used. The presence of colonies indicates an interaction between the test segments. Each co-transformation was performed 5–15 times.

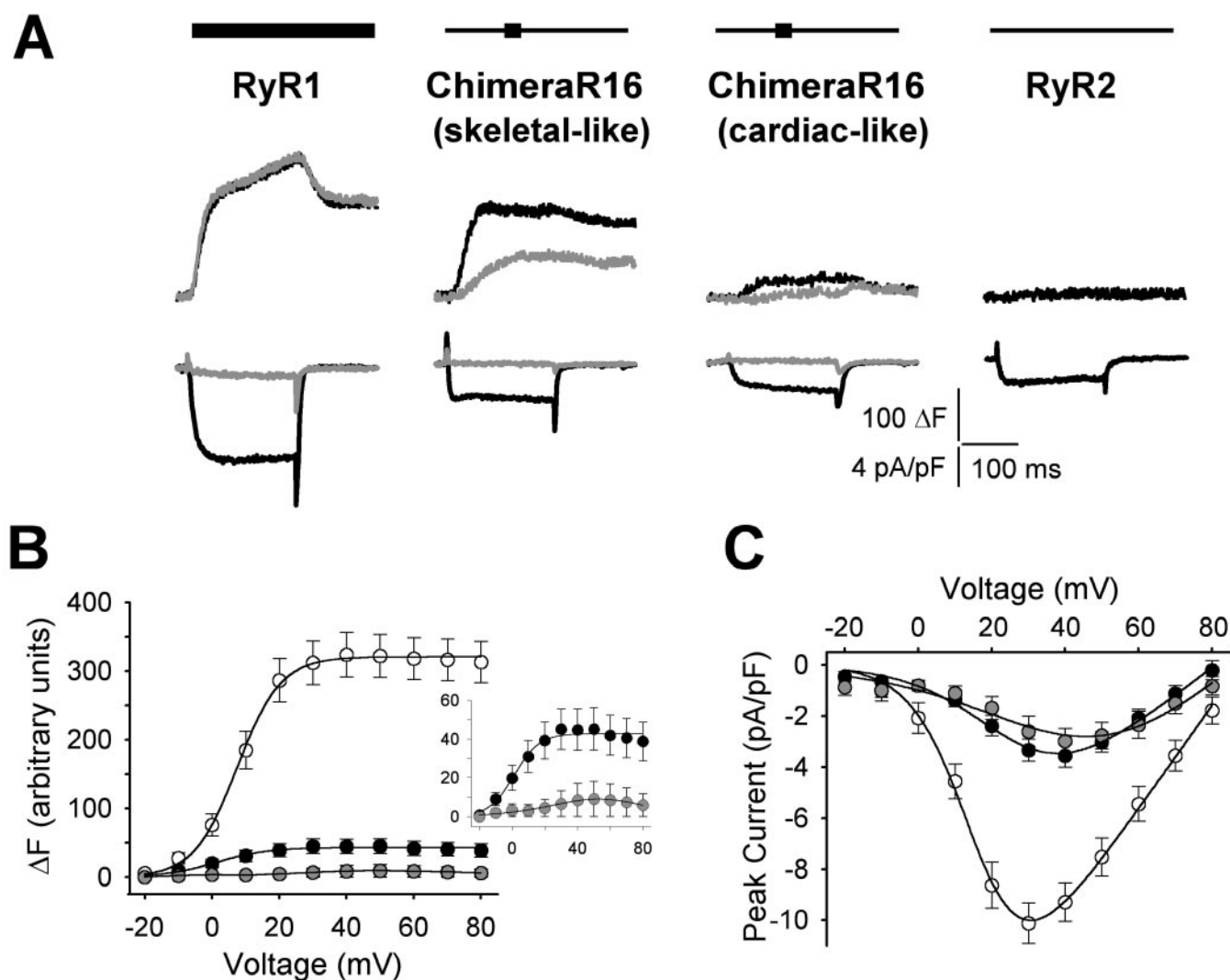
required incubation of the plates for 7–10 days. This indicates that the interaction is quite weak, because colonies appeared within 2–4 days (data not shown) for yeast co-transformed with  $\alpha_{1D}$  and  $\beta_{1D}$ , which are responsible for the strong binding of the  $\beta$  subunit to  $\alpha_{1S}$  (31). The *lacZ* reporter gene (which encodes  $\beta$ -galactosidase) yielded similar results; a blue reaction product, indicating expression of  $\beta$ -galactosidase, was observed for yeast containing s53 and sR16 in four of four separate transformations. To ensure that the yeast two-hybrid positive result for the s53  $\times$  sR16 interaction was independent of the GAL4 segments of the fusion proteins, we constructed the mirror clones with s53 inserted into the GAL4 activation domain vector and sR16 inserted into the GAL4 binding domain vector. An interaction was observed in three of four transformations performed with this pair of clones. The specificity of the interaction between s53 and sR16 was also tested by replacing them with c53 and cR16, respectively. The colonies were not observed for c53 when co-transformed with any of the RyR1 segments ( $n = 5$ ) but were occasionally seen on test plates streaked with yeast bearing cR16 and s53 (5 of 14 transformations). Thus, s53 is also able to interact with the cR16 region of RyR2, albeit less efficiently than with the sR16 region of RyR1.

**ChimeraR16 Mediates Weak Skeletal-type EC Coupling**—To determine whether there was a physiological correlate of the yeast interaction between s53 and sR16, we constructed chimeraR16, a chimeric skeletal-cardiac ryanodine receptor. ChimeraR16 had RyR2 sequence except for replacement of residues 1817–2118 by the corresponding region of RyR1 (1837–2154). Thus, chimeraR16 contained all but the final 14 residues (2155–2168) of sR16, which were omitted for cloning reasons and which, because RyR1 and RyR2 are highly conserved in this region, resulted in only three amino acid changes (E2157A, C2158S, and I2167S, using RyR1 numbering). RyR1, chimeraR16, and RyR2 were expressed in dyspedic myotubes (which lack endogenous RyR1). Whole cell patch clamping in combination with the  $\text{Ca}^{2+}$  indicator Fluo-3 was used for the simultaneous measurement of  $\text{Ca}^{2+}$  currents and  $\text{Ca}^{2+}$  transients resulting from intracellular release. Because skeletal EC coupling is defined as being independent of entry of  $\text{Ca}^{2+}$  through the DHPR, we first tested chimeraR16 for the ability to participate in EC coupling when  $\text{Ca}^{2+}$  current was blocked by 0.1 mM  $\text{Cd}^{2+}$  and 0.5 mM  $\text{La}^{3+}$  (10 of 22 cells expressing chimeraR16 were tested in this fashion). Fig. 2A illustrates representative  $\text{Ca}^{2+}$  currents and transients recorded from myotubes express-

ing RyR1, chimeraR16, or RyR2. For RyR1, large  $\text{Ca}^{2+}$  currents and transients were observed, and the transient was little affected when the current was blocked by  $\text{Cd}^{2+}$  and  $\text{La}^{3+}$  (gray traces). In contrast,  $\text{Ca}^{2+}$  current was small in cells expressing RyR2, and little or no transient was present even when the current was not blocked. The behavior of myotubes expressing chimeraR16 was intermediate between RyR1 and RyR2. Most cells expressing chimeraR16 (8 of 10) had depolarization-induced  $\text{Ca}^{2+}$  transients that were reduced in amplitude, but not abolished, by blockade of current with  $\text{Cd}^{2+}$  and  $\text{La}^{3+}$  (for these 8 cells, the amplitude of the peak transient in the presence of  $\text{Cd}^{2+}$  and  $\text{La}^{3+}$  was  $67.2 \pm 8\%$  that of control). Other cells (2 of 10) had much smaller transients that were eliminated by  $\text{Cd}^{2+}$  and  $\text{La}^{3+}$ .

A second defining feature of skeletal-type EC coupling is that the amplitude of the  $\text{Ca}^{2+}$  transient does not have a voltage dependence mirroring that of the peak  $\text{Ca}^{2+}$  current;  $\text{Ca}^{2+}$  transient amplitude in skeletal muscle has a sigmoidal rather than a bell-shaped voltage dependence. Fig. 2B illustrates the voltage dependence of intracellular  $\text{Ca}^{2+}$  transients for each of the three RyR constructs measured in the standard extracellular solution containing 10 mM  $\text{Ca}^{2+}$ . For RyR1, the transient showed a sigmoidal dependence on voltage, unlike peak  $\text{Ca}^{2+}$  currents, which had a U-shaped voltage dependence (Fig. 2C). This behavior is consistent with skeletal-type EC coupling, which is independent of the entry of extracellular  $\text{Ca}^{2+}$  (5). The voltage dependence of the transients for chimeraR16 suggested that they were partially dependent on  $\text{Ca}^{2+}$  entry (because they were maximal at about the same potential at which inward current peaked) but that they also contained a component of skeletal-type coupling because the transients were still appreciable at +80 mV (Fig. 2B, inset) and persisted after addition of  $\text{Cd}^{2+}$  and  $\text{La}^{3+}$ , as described above. Thus, the presence of skeletal sequence in chimeraR16 clearly altered the properties of intracellular  $\text{Ca}^{2+}$  release, causing it to be partially independent of  $\text{Ca}^{2+}$  entry. Another indication of the importance of the skeletal sequence in chimeraR16 was that the average amplitude of the peak transient was substantially larger than for RyR2; at +30 mV, the peak  $\text{Ca}^{2+}$  transient for cells expressing chimeraR16 averaged  $45.0 \pm 10.4$  fluorescence units ( $n = 22$ ), which was significantly larger ( $p < 0.025$ ) than in cells expressing RyR2 ( $6.6 \pm 6.6$ ,  $n = 10$ ), although significantly smaller ( $p < 10^{-8}$ ) than for cells expressing RyR1 ( $312 \pm 31.7$ ,  $n = 29$ ).

As a separate assay of the ability of chimeraR16 to partici-



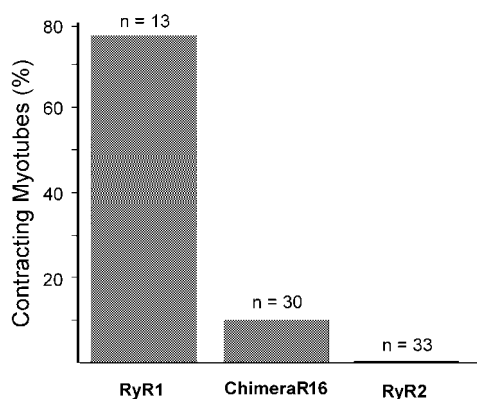
**FIG. 2. ChimeraR16 participates in signaling with  $\alpha_{1S}$ .** *A*, representative  $Ca^{2+}$  fluorescence signals (*middle*) and L-type  $Ca^{2+}$  currents (*bottom*) recorded simultaneously from dyspedic myotubes expressing RyR1, chimeraR16, or RyR2 (illustrated schematically at *top*; *thick lines* indicate RyR1 sequence, and *thin lines* indicate RyR2 sequence). The cells were bathed in either standard extracellular solution containing 10 mM  $Ca^{2+}$  (*black lines*) or standard solution with the addition of 0.5 mM  $Cd^{2+}$  and 0.1 mM  $La^{3+}$  (*gray lines*). The fluorescent calcium indicator Fluo-3 was used to monitor intracellular  $Ca^{2+}$  transients. The illustrated currents and transients were elicited by test depolarizations to +40 mV. *B*, voltage dependence of intracellular  $Ca^{2+}$  transients recorded from dyspedic myotubes expressing RyR1 (*open circles*,  $n = 29$ ), chimeraR16 (*black circles*,  $n = 22$ ), or RyR2 (*gray circles*,  $n = 10$ ) in standard extracellular solution. The amplitudes of the  $Ca^{2+}$  transients in cells expressing RyR1 have sigmoidal voltage dependence and plateau at about +40 mV. Unlike for RyR1,  $Ca^{2+}$  transient amplitudes for chimeraR16 sag somewhat for depolarizations beyond +40 mV but not nearly to the extent shown by RyR2 (*inset*). *C*, average  $Ca^{2+}$  current densities as a function of test potential are compared for dyspedic myotubes expressing RyR1 (*open circles*,  $n = 35$ ), chimeraR16 (*black circles*,  $n = 33$ ), or RyR2 (*gray circles*,  $n = 10$ ).  $Ca^{2+}$  current densities for cells expressing chimeraR16 are smaller than for cells expressing RyR1 but larger than those in cells expressing RyR2.

pate in skeletal-type EC coupling, we determined whether electrical stimulation could elicit contractions in the absence of  $Ca^{2+}$  entry through  $\alpha_{1S}$ . Electrically evoked contractions were observed in almost 80% of dyspedic myotubes expressing RyR1, whereas none were observed in cells expressing RyR2 (Fig. 3). Contractions were seen in 3 of 30 cells expressing chimeraR16, consistent with the presence in such cells of  $Ca^{2+}$  transients that were resistant to  $Cd^{2+}/La^{3+}$  (Fig. 2A). The much higher percentage of  $Cd^{2+}/La^{3+}$ -resistant  $Ca^{2+}$  transients in chimeraR16-expressing cells (8 of 10 cells tested) probably represents  $Ca^{2+}$  release fluxes that are too small to elicit contraction in response to a brief stimulus but that are still sufficiently large to produce measurable transients in response to a prolonged depolarization.

**Lack of Retrograde Signaling by ChimeraR16**—To determine the ability of chimeraR16 to enhance  $Ca^{2+}$  current via  $\alpha_{1S}$  (retrograde signaling) (6), we measured  $Ca^{2+}$  current density in dyspedic myotubes expressing RyR1, chimeraR16, or RyR2.

As shown in Fig. 2C,  $Ca^{2+}$  current density in myotubes expressing chimeraR16 was much smaller than in cells expressing RyR1 and only a little larger than in cells expressing RyR2, which was previously shown (6) not to produce retrograde enhancement of  $Ca^{2+}$  current. On average, the maximal peak  $Ca^{2+}$  current density in cells expressing chimeraR16 was  $4.04 \pm 0.44$  pA/picofarad ( $n = 33$ ), a value significantly ( $p < 10^{-10}$ ) smaller than that in cells expressing RyR1 ( $10.02 \pm 0.79$  pA/picofarad,  $n = 35$ ) and not statistically different ( $p$  value of 0.287) from that in cells expressing RyR2 ( $3.11 \pm 0.56$  pA/picofarad,  $n = 18$ ). Thus, chimeraR16 appears unable to carry out retrograde signaling to  $\alpha_{1S}$ .

**The R16 Region Is Not Required for Bi-directional Signaling with  $\alpha_{1S}$** —The experiments described above indicated that the R16 region of RyR1 is sufficient for weak EC coupling. To determine whether this region is necessary for signaling, we constructed chimeraR16-rev, in which RyR2 residues 1637–2118 replaced residues 1645–2154 within RyR1. Thus, chime-



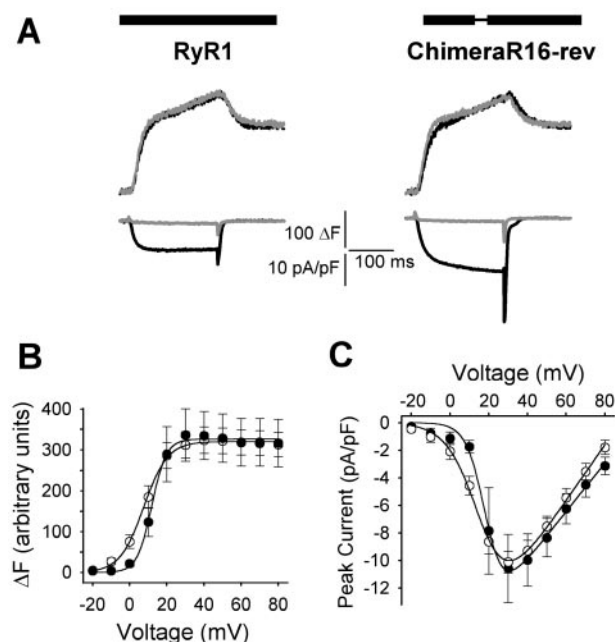
**FIG. 3. Some dyspedic myotubes expressing chimeraR16 contract in response to electrical stimulation in the absence of extracellular  $\text{Ca}^{2+}$ .** Dyspedic myotubes expressing the indicated construct were bathed in a  $\text{Ca}^{2+}$ -free Ringer's solution and were assayed for contraction in response to extracellular electrical stimulation. Electrically induced contractions were not observed in any of the RyR2-expressing myotubes (0 of 33 cells) but were observed in 3 of 30 cells expressing chimeraR16 and in 10 of 13 cells expressing RyR1.

raR16-rev lacks the RyR1 sequence (1837–2154) present in chimeraR16 as well adjacent RyR1 sequence region toward the amino terminus. If the sR16 region were necessary for bi-directional signaling, one would expect signaling to be lacking in cells expressing chimeraR16-rev. In fact, both  $\text{Ca}^{2+}$  transients and  $\text{Ca}^{2+}$  currents in dyspedic myotubes expressing chimeraR16-rev were similar to those in cells expressing RyR1 (Fig. 4). Moreover, chimeraR16-rev did not differ from RyR1 in its ability to mediate skeletal-type EC coupling, as indicated by electrically evoked contractions in the absence of  $\text{Ca}^{2+}$  entry through  $\alpha_{1S}$ . Specifically, 31 of 38 myotubes expressing chimeraR16-rev contracted in response to electrical stimulation, a proportion similar to that for RyR1-expressing myotubes (Fig. 3). Thus, the R16 region is not necessary for skeletal-type EC coupling or retrograde signaling.

#### DISCUSSION

In this paper, we have identified a region of RyR1 ("sR16," residues 1837–2168) that interacts with a portion of the  $\alpha_{1S}$  II–III loop ("s53," residues 720–765) in yeast two-hybrid assays. A weaker interaction occurred between the corresponding region of RyR2 ("cR16," residues 1817–2142) and s53. However, sR16 did not interact either with an upstream portion of the  $\alpha_{1S}$  II–III loop ("s31," residues 666–709) or with the portion of the  $\alpha_{1C}$  II–III loop ("c53," residues 850–897) that corresponds to s53. Expression in dyspedic myotubes of chimeraR16, a chimeric ryanodine receptor in which the cR16 region of RyR2 was replaced by sR16, was used to investigate the functional significance of the yeast interaction. In the absence of  $\text{Ca}^{2+}$  entry through  $\alpha_{1S}$ , both intracellular  $\text{Ca}^{2+}$  transients and depolarization-induced contractions revealed that chimeraR16 can mediate weak skeletal-type EC coupling. These functional results, together with the yeast two-hybrid experiments, are consistent with the idea that physical contact between the sR16 region of RyR1 and the s53 region of  $\alpha_{1S}$  may be involved in signaling between these two proteins.

To determine whether sR16 is essential for bi-directional signaling, we constructed chimeraR16-rev in which a region of RyR1 that includes sR16 was replaced by RyR2 sequence. ChimeraR16-rev retained almost normal ability to mediate bi-directional signaling. Thus, it is clear that signaling does not depend upon the presence of RyR1 sequence in the D3 domain, which is highly divergent between RyR1 and RyR2 (32) and is contained in the sR16/cR16 region. The ability of chimeraR16-rev to participate in bi-directional signaling also suggests that



**FIG. 4. Dyspedic myotubes expressing chimeraR16-rev mediate bi-directional coupling that does not differ from that of myotubes expressing RyR1.** A, schematic illustrations of the RyRs used to assay bi-directional signaling (*top*). A *thick line* indicates RyR1 sequence, and a *thin line* indicates RyR2 sequence. Representative  $\text{Ca}^{2+}$  transients (*middle*) and  $\text{Ca}^{2+}$  currents (*bottom*) were elicited by depolarizations to either +40 mV (RyR1) or +50 mV (chimeraR16-rev) before (*black*) and after (*gray*) addition of 0.5 mM  $\text{Cd}^{2+}$  and 0.1 mM  $\text{La}^{3+}$  to the bathing medium. B, average voltage dependence and amplitude of intracellular  $\text{Ca}^{2+}$  transients recorded from dyspedic myotubes expressing RyR1 (*open circles*,  $n = 27$ ) or chimeraR16-rev (*filled circles*,  $n = 6$ ) in normal extracellular solution. C, the voltage dependence and the average amplitude of the peak  $\text{Ca}^{2+}$  current density of dyspedic myotubes expressing RyR1 (*open circles*,  $n = 27$ ) or chimeraR16-rev (*filled circles*,  $n = 6$ ) do not differ significantly.

the interaction between the s53 and sR16 regions is not the only site of coupling between  $\alpha_{1S}$  and RyR1. However, the ability of chimeraR16-rev to behave almost like RyR1 does not imply that there is no functional role for binding between  $\alpha_{1S}$  and the sR16 region of RyR1 because the cR16 region of RyR2 may also be able to bind to  $\alpha_{1S}$ , as suggested by the yeast two-hybrid experiments (Fig. 1).

The yeast two-hybrid results reinforce previous findings with  $\alpha_{1S}$  chimeras. First, s31 does not bind in two-hybrid experiments to the regions of RyR1 tested here, consistent with observation that bi-directional signaling does not depend on the presence of skeletal sequence in this region (7, 8, 19, 20). Second, s53 binds to sR16, but c53 does not. This is consistent with observations of chimeric  $\alpha_1$  channels demonstrating that the s53 region is essential for bi-directional signaling (7, 8) and that this signaling is lost when s53 is replaced by c53. Thus, s53 may be one site at which  $\alpha_{1S}$  contacts RyR1 (in the R16 region), consistent with a direct allosteric activation mechanism for coupling between  $\alpha_{1S}$  and RyR1.

It is important to consider limitations of the yeast two-hybrid method, which indicate that a failure of interaction cannot be interpreted to mean that the corresponding protein regions do not interact *in vivo*. First, our use of segments of RyR1 with only a small amount of overlap might have interrupted an interaction motif. More fundamentally, there is an inherent size limitation of segments that can be explored with the two-hybrid method (about 300 residues). These ~300 residue segments might have failed to achieve a native conformation or been inappropriately expressed as yeast fusion proteins. For all these reasons, we might have missed interactions between  $\alpha_{1S}$

and segments of RyR1 outside of sR16 or may have missed a stronger interaction that was interrupted by separation of sR16 from its flanking regions. Finally, the reactivity in yeast between sR16 and s53 is consistent with but does not prove that these residues contact one another in living cells. However, the physiological results clearly show that both regions are important for the functional interaction between RyR1 and  $\alpha_{1S}$ . Additionally, the physiological results show that coupling between RyR1 and  $\alpha_{1S}$  cannot depend solely on the sR16 region.

A number of observations suggest that multiple segments of the primary sequence of both  $\alpha_{1S}$  and RyR1 may be involved in bi-directional signaling. First, Nakai *et al.* (9) found that two different RyR chimeras were able to support signaling interactions between  $\alpha_{1S}$  and RyR1. These chimeras contained non-overlapping RyR1 sequence (residues 1635–2636 or 2659–3720) in a background of RyR2. Second, we have shown here that chimeraR16 supports weak signaling and that stronger signaling is supported by chimeraR16-rev, suggesting that regions of RyR1 in addition to sR16 contribute to signaling. Third, biochemical experiments have shown effects on RyR1 function by peptides corresponding both to the II–III loop (12–18) and the carboxyl tail (33) of  $\alpha_{1S}$ . Moreover, relatively strong binding interactions have been found between the  $\alpha_{1S}$  II–III loop and intact RyR1 (34) as well as between both the I–II and II–III loops of  $\alpha_{1S}$  and a fusion protein corresponding to RyR1 residues 951–1112 (10, 11). Interestingly, these latter experiments failed to reveal binding of the  $\alpha_{1S}$  II–III loop to sR16-containing fusion peptides. Perhaps this binding is so weak that it can be detected only with the yeast two-hybrid technique, which is more sensitive than more standard binding assays. The explanation is less obvious, however, for why the yeast two-hybrid analysis has failed to reveal the strong binding interactions found in the biochemical experiments. In particular, no strong interactions were observed either in the present work or in earlier yeast two-hybrid experiments in which a skeletal muscle cDNA library was screened with the amino-terminal, I–II, II–III, or III–IV loops of  $\alpha_{1S}$  (35). This may result from a distinct disadvantage of the yeast two-hybrid system, that the size of test fragments is limited to a maximum of about 300 residues and that smaller test fragments appear to reconstitute activity of the GAL4 transcription factor more efficiently than do larger fragments (35), which is particularly problematic for studies of a protein as large as the RyR. Because it is not possible to screen larger regions of primary sequence for interaction using the two-hybrid system, interaction domains consisting of noncontiguous primary sequence could be missed using the two-hybrid approach. Nonetheless, the method has provided the first identification of a domain that may participate in both direct interaction and functional coupling.

Currently, a scarcity of information about how the primary sequence of RyRs is arranged in three dimensions hampers both the functional analyses of RyR chimeras and the biochemical analyses of potential interactions between  $\alpha_{1S}$  and RyR1. For example, we do not know whether either the sR16 region or the region identified by Leong and MacLennan (10) (residues 951–1112) is exposed to the cytoplasmic surface of RyR1 that abuts transverse tubules. Obviously, a demonstration that  $\alpha_{1S}$  binds to a segment of RyR1 is unlikely to be physiologically significant if that segment is not accessible to  $\alpha_{1S}$  *in vivo*. Just

as importantly, we do not know whether the primary sequence of sR16 (for example) contributes to one or several structural domains of the RyR1, a major limitation in interpreting the functional behavior of the R16 chimera. It is also possible that sR16 is just one part of a larger domain involved in interaction with  $\alpha_{1S}$ . However, even though it is not currently possible to produce RyR chimeras on the basis of a known relationship between primary sequence and three-dimensional structure, the chimeras remain a valuable tool for testing the functional importance of interactions between  $\alpha_{1S}$  and RyR1 that have been identified *in vitro*.

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